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L. Harper

PI - Signature Date

Table of Contents

Appendix to the Final Report for DAMD17-94-J-4399
"The role of p21CIP1 in Breast Cancer"

Introduction

The final report for my award, DAMD17-94-J-4399 was prematurely submitted in the Fall of 1998 and approved in 1999. This report was changed from a final to an annual by the Office of the Deputy Chief of Staff for Information Management due to the fact that we had asked for and was granted a one year no cost extension. This extension allowed us to finish up our work on the p21 knockout mouse, as summarized below, and led to an additional publication. The attached material is submitted as an appendices to our report submitted in the Fall of 1998. The Fall 1998 report should be reclassified as a final and this award closed. All pertinent information relating to a final report can be found in the Fall 1998 report.

Analysis of the role of Cdk inhibitors in development and transformation

My laboratory has been collaborating with the laboratory of Dr. Steve Elledge (Co-Principal Investigator) since we discovered p21 in 1993. Our major goal in this work has been to understand the role of p21 in development and transformation. The finding by our lab and other labs that p21 is a member of a protein family containing p27 and p57 was unforeseen at the time the initial grant was written in 1993. Studies during this period have revealed that there is likely to be extensive functional overlap between different members of the CKI family. This is amplified by the extensive cell type specificity observed with the expression of various CKIs.

The question of functional redundancy is raised most profoundly by studies of knockout mice where one particular CKI may fulfill the function of the CKI lost by directed deletion of the gene. For example, since p21 knockout mice have no obvious developmental phenotype, it was possible that other CKIs (p27 or p57) were up-regulated in response to p21 loss and that they perform any essential functions of p21 in vivo. However, in our previous study, we were unable to find any alterations in the expression of p27 or p57 in p21 knockout mice (Deng et al., 1995). In a separate study, we have generated mice lacking p57 (Zhang et al.1997). We examined whether mice lacking p57 compensated for its loss by inducing either p21 or p57. We found no change in the levels of p21 in either muscle or kidney by immunoblotting. While p27 levels were also unaltered in kidney, there was a slight (50%) increase in p27 levels in p57 -deficient mice.

In order to examine the question of redundancy in greater detail, we cross p21-/- mice to p57-deficient mice to create double knockout mice. The expectation is that specific tissues which employ redundant utilization of multiple inhibitors might give rise to more severe phenotypes than the single mutants alone. In a parallel series of studies we have found that p27 and p57 collaborate to control cell cycle exit in the differentiating lens (Zhang et al., 1998, see attached paper) Thus, there are clear cases where CKIs act

redundantly. In the 1998 Progress Report, we presented evidence that p21 and p57 also act redundantly to control development in the lungs and also in the skeletal system.

Upon further analysis of these mice during the extension period of the grant, we have found overt defects in muscle development as well. This work, along with an analysis of lung and skeletal defects discussed in the 1998 report were published this year in *Genes and Development* (Zhang et al., 1999; see attached Reprint). In brief, mice lacking both p21 and p57 fail to form myotubes, display increased proliferation and apoptotic rates of myoblasts, and display endoreduplication in residual myotubes. This point of arrest during muscle development is identical to that of mice lacking the myogenic transcription factor myogenin, indicating a role for cell-cycle exit in myogenin function. Expression of myogenin, p21, and p57 is parallel and independent, and in response to differentiation signals, these proteins are coordinately regulated to trigger both cell cycle exit and a dependent muscle-specific program of gene expression to initiate myoblast terminal differentiation and muscle development. Defects in muscle development correlate with effects on Rb phosphorylation, due to combinatorial loss of the two key Cdk inhibitors in these cells, p21 and p57. A model was developed to explain the differentiation trigger controlling myoblast terminal differentiation (See Figure 7 in Zhang et al., 1999). The key finding here is that cell cycle exit is required for myogenin function and not MyoD as previously thought.

Taken together, our studies on Cdk inhibitors and p21 in particular have provided insights into the mechanisms used by cells to control proliferation in distinct tissues. In addition, we have learned of the important role of p21 in the p53-dependent DNA damage response pathway. This concludes our studies funded by DAMD17-94-J-4399. I would also like to point that as a result of this funding, a talented post-doc, Dr. Pumin Zhang, was able to obtain a junior faculty position and is planning to continue this analysis of Cdks in development in his own lab.

Publications funded by DAMD17-94-J-4399

Papers indicated by * are attached. All others were provided witht he previous reports.

*Zhang, P., Wong, C., Liu, D., Finegold, M., Harper, J.W., and Elledge, S.J. (1998) p21CIP1 and p57KIP2 control muscle differentiation at the myogenin step. ***Genes and Development*** 13, 213-214.

*Zhang, P., Wong, C., DePhinho, R.A., Harper, J.W., and Elledge, S.J. (1998) Cooperation between the Cdk inhibitors p27KIP1 and p57KIP2 in the control of tissue growth and development. ***Genes and Development*** 12, 3162-3167.

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$p21^{CIP1}$ and $p57^{KIP2}$ control muscle differentiation at the myogenin step

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Cell-cycle arrest is thought to be required for differentiation of muscle cells. However, the molecules controlling cell-cycle exit and the differentiation step(s) dependent on cell-cycle arrest are poorly understood. Here we show that two Cdk inhibitors, $p21^{CIP1}$ and $p57^{KIP2}$, redundantly control differentiation of skeletal muscle and alveoli in the lungs. Mice lacking both p21 and p57 fail to form myotubes, display increased proliferation and apoptotic rates of myoblasts, and display endoreplication in residual myotubes. This point of arrest during muscle development is identical to that of mice lacking the myogenic transcription factor myogenin, indicating a role for cell-cycle exit in *myogenin* function. Expression of myogenin, p21, and p57 is parallel but independent, and in response to differentiation signals, these proteins are coordinately regulated to trigger both cell-cycle exit and a dependent muscle-specific program of gene expression to initiate myoblast terminal differentiation and muscle formation.

[*Key Words*: Muscle cell differentation; cell-cycle arrest; myogenin; Cdk inhibitors]

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Embryonic development is a complex process that requires precise spatial and temporal control of cell proliferation coordinated together with differentiation, morphogenesis, and pattern formation. Cell proliferation in the embryo is controlled by an intricate network of signal transduction pathways that integrate growth regulatory signals through regulation of cyclin-dependent kinases (Cdks), a family of enzymes that catalyze events required for cell-cycle transitions. Primary targets for this regulation are the G_1 cyclin/Cdk complexes cyclin D/Cdk4 and cyclin E/Cdk2, which cooperate to control the $G_1 \rightarrow S$ transition through phosphorylation and inactivation of the retinoblastoma (Rb) protein. Among other functions, Rb acts as a transcriptional repressor of E2F-regulated genes important for cell proliferation (Weinberg 1995). A large number of regulatory mechanisms exist to modulate Cdk activity, reflecting the complexity of the signaling pathways involved and the necessity to precisely control proliferation for the development of an organism. A particularly versatile mechanism for developmental control is the inhibition of Cdks by cyclin-dependent kinase inhibitors (CKIs), of which there are two families: the $p16^{INK4a}$ family, including p15, p16, p18, and p19; and the $p21^{CIP1/WAF1}$ family, including p21, p27, and p57 (Harper and Elledge 1996). The p16 family specifically inhibits Cdk4 and Cdk6, whereas the p21 family inhibits all Cdks involved in G_1/S transition. The roles of these CKIs in development and in cancer have been revealed through targeted gene inactivation in mice. Although CKIs show striking tissue-specific patterns of expression during development, surprisingly only p57 loss has been shown to have a significant role in multiple tissues during embryonic development, suggesting that the other inhibitors are either not required or are redundant. Loss of p57 in humans results in the complex overgrowth and cancer predisposition disease Beckwith–Wiedemann syndrome (Zhang et al. 1997).

A variety of studies have indicated that cells must exit the cell cycle to terminally differentiate. The most revealing of these come from the analysis of skeletal muscle development. Skeletal muscle development is controlled by a group of basic helix–loop–helix (bHLH) transcription factors, including MyoD, Myf5, myogenin, and Mrf4, each of which is capable of forcing nonmuscle cells to adopt skeletal muscle phenotypes when expressed ectopically (Olson and Klein 1994). Analysis of mice lacking bHLH myogenic factors has revealed a genetic hierarchy in which MyoD and Myf5 play redundant roles in specifying muscle lineage (the generation of myoblasts); myogenin directly controls the differentiation process (the formation of myotubes); and Mrf4 is thought to be involved in the maturation of myotubes. Early studies using cultured myoblasts revealed that cell-cycle exit and differentiation are coupled (Bischoff and Holtzer 1969; Nadal-Ginard 1978; Clegg et al. 1987).

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Furthermore, MyoD ectopically expressed in fibroblasts fails to function if these cells are also provided proliferative stimulation in the form of additional expression of cyclin D (Rao et al. 1994; Skapek et al. 1995), cyclin A, or cyclin E (Skapek et al. 1996). Additional connections between cell-cycle exit and differentiation have been established through analysis of the role of Rb in skeletal muscle differentiation. These include observations that viral oncogenes capable of inactivating Rb, such as T antigen and E1A, can interfere with muscle differentiation (Fogel and Defendi 1967; Yaffe and Gershon 1967; Graessmann et al. 1973; Endo and Nadal-Ginard 1989; Taylor et al. 1993; Crescenzi et al. 1995) and that cells lacking Rb fail to properly differentiate in vitro (Gu et al. 1993; Novitch et al. 1996). Although *Rb*-deficient mice display an apparently normal musculature (Clarke et al. 1992; Jacks et al. 1992; Lee et al. 1992), the early lethality of these mice [before embryonic day 14.5 (E14.5)]has precluded analysis of the role of Rb in secondary myogenesis when the majority of skeletal muscles are formed (Kelly 1983). Recently, Zacksenhaus et al. (1996) reported that $Rb^{-/-}$ embryos can be rescued to birth by the low-level expression of an *Rb* transgene, and these embryos show skeletal muscle defects. Taken together, this body of evidence points to an important role for Rb in control of cell-cycle exit and differentiation in myogenesis.

The Rb protein acts as a switch operated by cell-cycle Cdk machinery to control cell-cycle entry and exit and is likely to be a critical target of Cdk regulation relevant to differentiation. However, it is not at all clear which component of the myogenic regulatory hierarchy requires Rb for its function. Rb has been shown to interact with MyoD in vitro (Gu et al. 1993), although the significance of the interaction is brought into question by the fact that early targets of MyoD transcription appear to be fully induced in the absence of Rb (Novitch et al. 1996). How Rb is activated to facilitate myogenic differentiation is currently not known. Although it is likely that Cdk inactivation is employed, other mechanisms such as increasing the activity of Rb specific phosphatases are also possible. Furthermore, assuming that Cdk inactivation is the mechanism, how this is achieved is not known.

Initial insights into how cell-cycle exit is achieved in vivo came from the observation that the CKI p21 is highly expressed in muscle and other terminally differentiating tissues in vivo (Parker et al. 1995) and in vitro (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995). Furthermore, cells ectopically expressing MyoD can induce p21 when stimulated to differentiate in vitro suggesting that p21 is a downstream target of MyoD. However, mice lacking p21 develop normally and fail to show muscle cell differentiation defects, bringing into question the role of p21 in muscle development (Deng et al. 1995). The absence of a role for p21 in skeletal muscle development could be explained by redundancy of the cell-cycle control mechanisms. We report such a redundant mechanism in this study by showing that mice lacking both $p21^{CIP1}$ and $p57^{KIP2}$ display severe defects in skeletal muscle development (and other tissues including lung); this indicates that these proteins cooperate as terminal effectors of signaling pathways that impinge on cell-cycle control and differentiation. Furthermore, the stage in muscle cell differentiation at which these double mutants arrest reveals the point at which cell-cycle exit is required and implicates myogenin function as the critical step requiring cell-cycle exit.

Results

Generation of mice lacking both $p21^{CIP1}$ and $p57^{KIP2}$

To generate mice lacking *p21* and *p57*, we crossed $p21^{+/-}$ $p57^{-p/+}$ females to $p21^{-/-}$ males. Animals inheriting the mutant *p57* allele from the mother have a *p57* null phenotype because imprinting renders the paternally inherited allele silent. Consistent with our previous report (Zhang et al. 1997), there were no live-born mice lacking either *p57* or both *p21* and *p57* functions (data not shown). However, E16.5 embryos of all genotypes were detected at Mendelian ratios. A substantial fraction of *p57–m/+* single mutant (30%) and $p21^{-/-}$ $p57^{-m/+}$ double mutant (65%) embryos die in utero due to placental failure (Table 1). Thus, loss of *p21* exacerbates the placental defects observed in $p57^{-m/+}$ mutants. The following phenotypic analysis on $p21^{-/-}$ $p57^{-m/+}$ double mutants was based on animals that were not affected by placental failures.

$p21^{-/-}$ $p57^{-m/+}$ *double mutants show altered lung development*

Histopathological examination of $p21^{-/-}$ $p57^{-m/+}$ mice revealed all of the phenotypes caused by *p57* loss alone (Yan et al. 1997; Zhang et al. 1997) and several novel phenotypes in tissues that are apparently unaffected in either of the single mutant animals. Unlike $p21^{-/-}$ or $p57^{+/-m}$ animals, the lungs of $p21^{-/-}$ $p57^{-m/+}$ animals were clearly defective, failing to fully differentiate distal air sacs, the ultimate functioning unit for gas exchange in lung tissue. The mammalian lung is composed of two types of tissues: an epithelium that lines all the airways from the trachea to alveoli, and a mesenchymal stroma that supports the epithelium. Lung development is divided into several periods. In the pseudoglandular period

Table 1. *Distribution of genotypes among embryos derived from a cross between $p21^{-/-}$ $p57^{+/+}$ males and $p21^{+/-}$ $p57^{+/-p}$ females*

Genotype	$p21^{+/-}$ $p57^{+/+}$	$p21^{-/-}$ $p57^{+/+}$	$p21^{+/-}$ $p57^{+/-m}$	$p21^{-/-}$ $p57^{+/-m}$
Number of embryos	26	24	20 (6)[a]	29 (19)[a]
Observed (%)	26	24	20	29
Expected (%)	25	25	25	25
Lethality (%)	0	0	30	65

[a]Number in parenthesis indicates the number of embryos that were already dead at the time of harvesting.

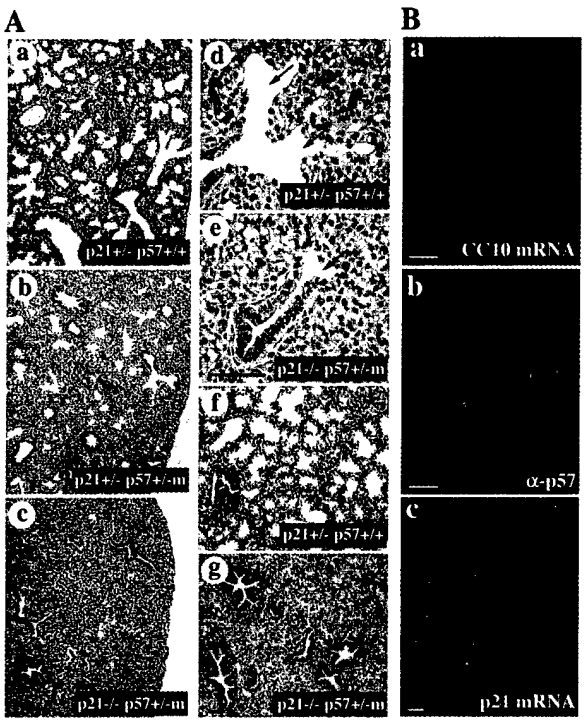

Figure 1. A block in the formation of primitive alveoli in the absence of both p21 and p57. (*A*) H&E-stained transverse sections of lungs derived from E16.5 (*a–e*) and from E18.5 embryos (*f,g*). (*B*) Expression of *p21* and *p57* in the lung of E18.5 embryos. (*a*) CC10 expression detected with in situ hybridization; (*b*) immunofluorescence staining of *p57*; (*c*) *p21* expression detected with in situ hybridization. Scale bars, 200 μm.

early during embryogenesis, the lung resembles an exocrine gland and consists of a complex of branching bronchial tubes that include the primary, secondary, segmental, and terminal bronchi and the bronchioles. This is followed by the canalicular period when respiratory bronchioles are formed. Each respiratory bronchiole is terminated in two or three thin-walled dilations termed terminal sacs or primitive alveoli. At E16.5, lungs from wild-type embryos display substantial formation of primitive alveoli manifested as open spaces on sections stained with hematoxylin and eosis (H&E) (Fig. 1A,a). In contrast, lungs from $p21^{-/-}$ $p57^{+/-m}$ animals are virtually devoid of open spaces (Fig. 1A,c). Under high magnification, it is evident that primitive alveoli do not develop in the double mutants (Fig. 1A, cf. d and e). This defect persists until birth (Fig. 1A, cf. f and g). Furthermore, there is a decrease in the size of the lumenal space of the bronchi and bronchioles in the double mutants. $p21^{+/-}$ $p57^{+/-m}$ lungs exhibit an intermediate phenotype between the wild-type and the double mutant with some primitive alveoli but fewer than in the wild type (Fig. 1A, cf. a, b, and c), indicating that a single *p21* gene is insufficient in the absence of p57.

To explore the cause of the lung defect, we examined the expression of both *p57* and *p21* in the developing lung. *p57* is highly expressed in bronchiole epithelium, mirroring that of CC10, a marker for that tissue. *p57* is expressed at lower levels in an undefined subset of lung mesenchymal cells and the epithelium lining of the terminal primitive alveoli (Fig. 1B). In contrast, *p21* is expressed throughout the lung. Despite high levels of expression of *p57* in the bronchiole epithelium, no significant abnormalities were detected in this tissue, and tissue-specific differentiation markers such as CC10 and SP-A, SP-B, and SP-C are expressed normally in double mutants (data not shown). Although the absence of air sac lumenal space gives the appearance of increased cellularity in the mutants, this is not the case. This is due to the fact that the lungs of the double mutant mice are smaller than the wild-type lungs (data not shown); thus, the total number of cells is approximately the same. Furthermore, the overall proliferation rates in the double mutant lung were not elevated as judged by BrdU pulse labeling nor was there an increase in apoptosis (data not

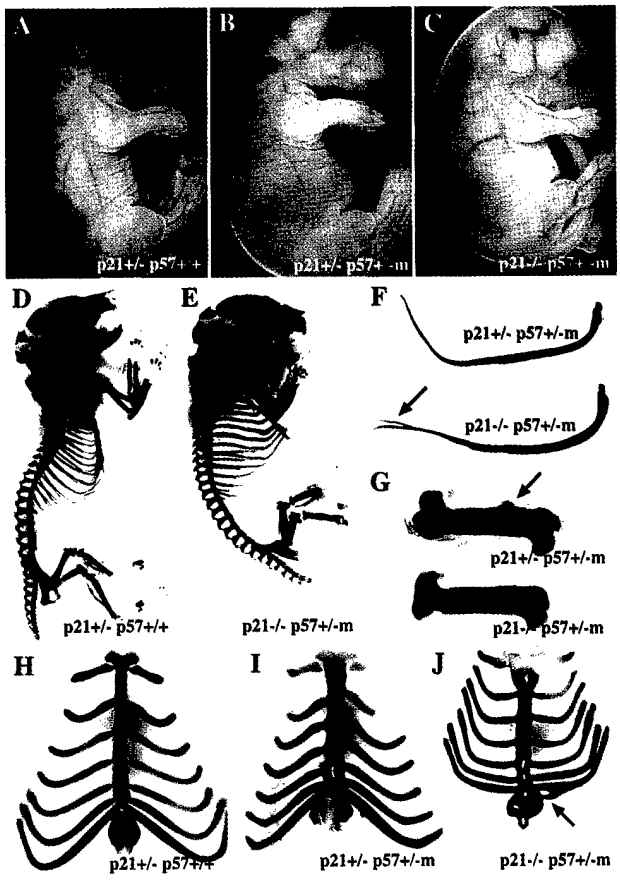

Figure 2. Skeletal defects in $p21^{-/-}$ $p57^{-m/+}$ mutants. (*A–C*) $p21^{-/-}$ $p57^{-m/+}$ embryos display altered posture. (*D,E*) Skeletons of E18.5 embryos stained with alcian blue to identify cartilage and alizarin red to identify ossified bone. (*F*) Bifurcation of the ninth rib (arrow) is observed in *p21–/–* $p57^{-m/+}$ embryos. (*G*) The femur of E18.5 embryos. The arrow indicates cartilage outgrowth. (*H–J*) Sternia and ribs of E18.5 embryos. Only 7 of the 13 ribs attach to the sternum. Note a bifurcation in the seventh rib in the double mutant (arrow in *J*). Embryos in *G–J* stained as in *D* and *E*.

shown). Thus, the defects in primitive alveoli formation in the absence of p21 and p57 is likely to result from subtle changes in the differentiation of either the epithelia or the mesenchymal stroma for which additional studies are required to delineate more precisely.

Skeleton defects in $p21^{-/-}$ $p57^{m/+}$ *double mutants*

The only phenotype of $p57^{+/-m}$ mice that is enhanced by loss of *p21* is the skeletal phenotype. Deletion of p57 alone causes delay in ossification and sternal fusion defects but no overall abnormality in the shape of the skeleton (Yan et al. 1997; Zhang et al. 1997). However, as shown in Figure 2, $p21^{-/-}$ $p57^{-m/+}$ double mutant embryos display a posture clearly distinct from those of wild-type and $p57^{-m/+}$ mutants (Fig. 2A–C). Skeleton staining revealed that double mutants (Fig. 2E) lack the spinal curvature seen in wild type (Fig. 2D) and $p57^{-m/+}$ single mutants (data not shown), which might stem from defects in musculature (see below). Rib cage shape in double mutant embryos is also abnormal (Fig. 2, cf. D and E). Bifurcation of ribs was observed in double mutants, usually of the ninth rib (Fig. 2F), although occasionally the seventh rib is also affected (Fig. 2J). The femurs of double mutants lack a cartilage outgrowth seen in either *p21* or *p57* single mutants or wild-type littermates (Fig. 2G; data not shown). The double mutants exhibited sternum fusion defects similar to those seen in *p57* single mutants (Zhang et al. 1997), but the sternum of double mutants is shorter than that of *p57* single mutants (Fig. 2H). The ribs of double mutants join the sternum at an angle of 90°C (Fig. 2J), whereas the ribs of wild type or *p57* single mutants join at an angle much less than 90°C (Fig. 2H). Both *p21* and *p57* have been found highly expressed in developing ribs (Zhang et al. 1997; and data not shown). However, it is difficult to distinguish autonomous versus nonautonomous roles of these two inhibitors in ribs, especially considering the fact that similar defects in the attachment of ribs to sternum

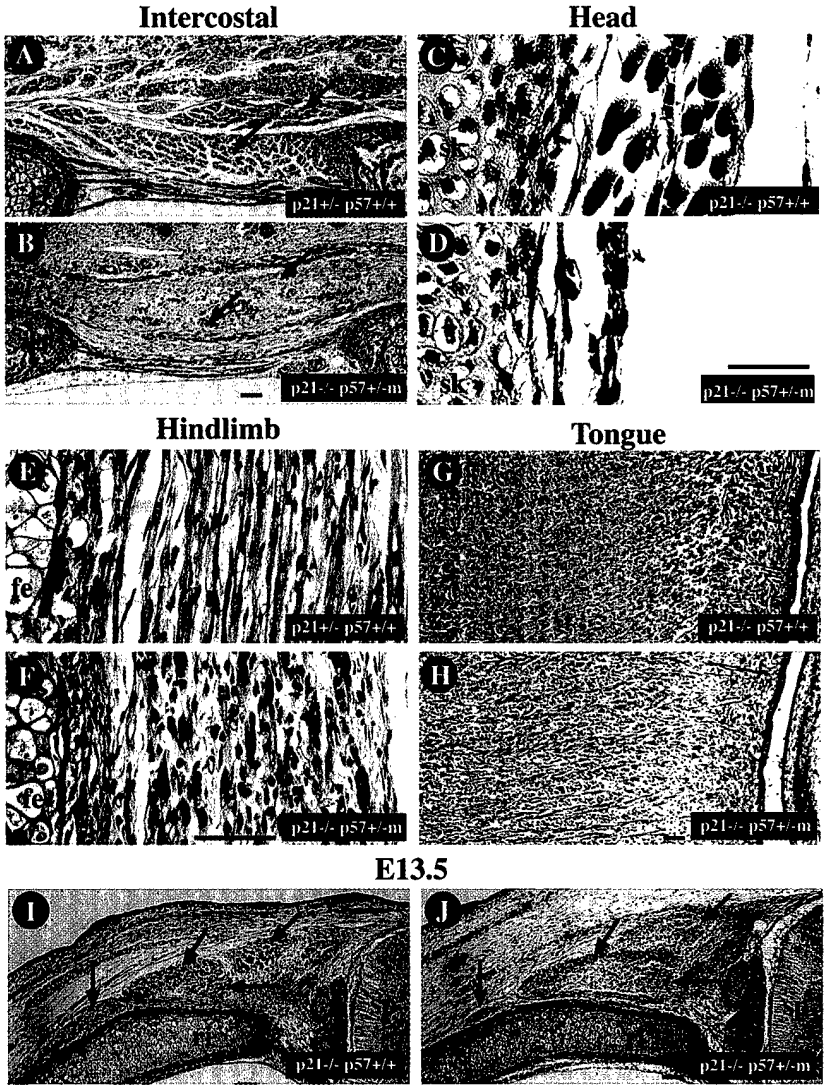

Figure 3. $p21^{-/-}$ $p57^{-m/+}$ double mutant mice display defects in skeletal muscle development. (*A–H*) H&E-stained transverse sections of E18.5 embryos. Arrows in *A* and *B* indicate intercostal skeletal muscles. (*I,J*) H&E-stained transverse sections of the chest region of E13.5 embryos. Arrows indicate various muscle groups. (fe) Femur; (ri) rib; (sk) skull; (sp) spinal cord. Scale bars, 200 μm.

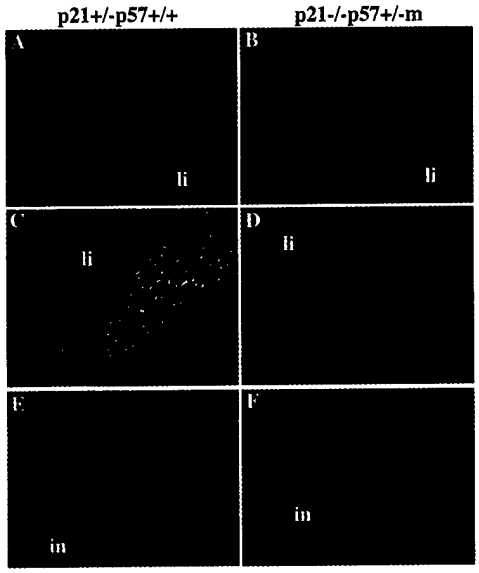

Figure 4. Diminished expression of MHC in the skeletal muscle of $p21^{-/-}$ $p57^{-m/+}$ double mutant embryos. Immunofluorescence staining was performed on transverse sections of E18.5 embryos of indicated genotypes with a monoclonal antibody against MHC. MHC expression was visualized with a Texas red-conjugated secondary antibody, and nuclei were stained with DAPI. (*A,B*) The root of the diaphragm; (*C,D*) the diaphragm; (*E,F*) the body wall. Genotypes are indicated above for each column. (in) Intestine; (li) liver.

are observed in mice lacking myogenin (Hasty et al. 1993; Nabeshima et al. 1993).

$p21^{-/-}$ $p57^{-m/+}$ *double mutants exhibit a profound defect in skeletal muscle*

Both p21 and p57 proteins are highly expressed in skeletal muscle, but neither single mutant animal showed significant muscle cell differentiation defects (Deng et al. 1995; Yan et al. 1997; Zhang et al. 1997). However, $p21^{-/-}$ $p57^{-m/+}$ double mutants exhibit profound defects in skeletal muscle development. We have found no significant difference in skeletal muscle development between $p21^{+/+}$ $p57^{+/-m}$ and $p21^{+/-}$ $p57^{+/-m}$ mice (data not shown), indicating that a single copy of the *p21* gene can fully support skeletal muscle development. As shown by H&E staining of transverse sections of E18.5 embryos, the intercostal muscle is greatly reduced in double mutants (Fig. 3, cf. A and B), and the head muscle is diminished (Fig. 3C,D). In the hind limb, numerous long myotubes are observed in $p21^{-/-}$ $p57^{+/+}$ embryos (Fig. 3E), but many fewer and shorter myotubes are present in double mutants (Fig. 3F). Defects in the tongue muscle were somewhat less severe, and double mutant animals exhibit slightly disorganized and less dense muscle mass when compared to $p21^{-/-}$ $p57^{+/+}$ animals (Fig. 3, cf. G and H).

The diaphragm and body wall muscles of double mutants are also severely diminished as demonstrated by immunofluorescence staining using a monoclonal antibody against myosin heavy chain (MHC). The root of the diaphragm in double mutants is much thinner and poorly stained by the antibody relative to the wild-type control (Fig. 4A,B). MHC staining was diminished in the

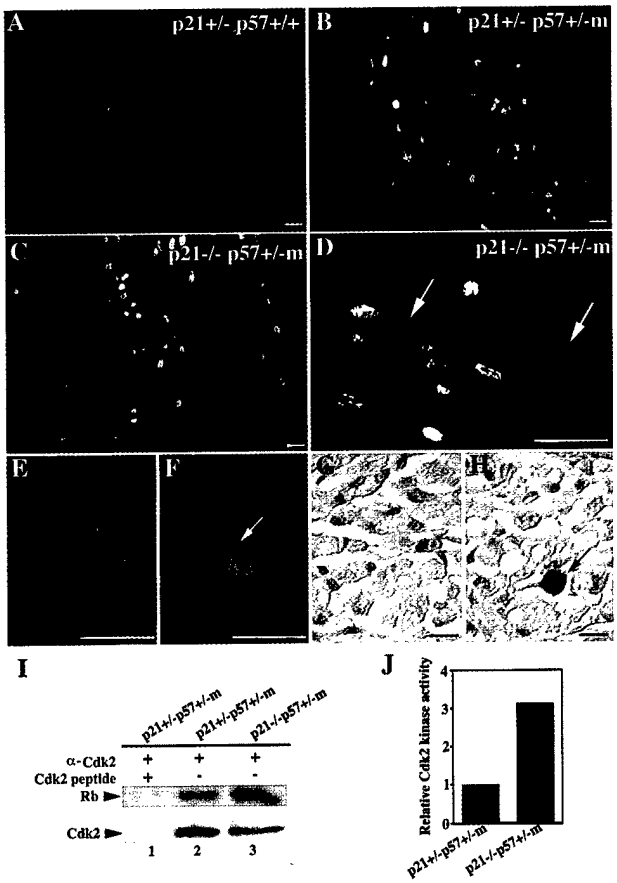

Figure 5. Increased rates of proliferation and apoptosis in *p21/p57* double mutants. (*A–D*) BrdU pulse-labeled cells in the intercostal region of E16.5 embryos were visualized by immunofluorescence staining with a monoclonal antibody against BrdU that was subsequently detected with a FITC-conjugated secondary antibody. Arrows indicate BrdU-positive nuclei in the residual myotubes that were revealed by background DAPI (blue) staining. (*E,F*) MHC immunofluorescence staining of transverse sections of the abdomen region of E18.5 $p21^{+/-}$ $p57^{+/+}$ (*E*) and $p21^{-/-}$ $p57^{+/-m}$ (*F*) embryos as in Fig. 4. Nuclei are visualized with DAPI staining (blue). Arrow indicates a giant nucleus. (*G,H*) TUNEL assays performed on transverse sections of the chest region of E16.5 $p21^{+/-}$ $p57^{+/+}$ (*G*) and $p21^{-/-}$ $p57^{+/-m}$ (*H*) embryos. Arrow indicates an apoptotic nucleus in the intercostal muscle. (*I*) The activity of CDK2 kinse immunoprecipitated from muscle extracts was assayed using Rb as a substrate by measuring the incorporation of [γ-^{32}P]ATP (*top*). The amount of CDK2 protein present in the immunoprecipitates monitored by Western blotting (*bottom*). (Lane *1*) Immunoprecipitation from $p21^{+/-}$ *p57+/-m* muscle extracts using anti-CDK2 antibody neutralized with excess competing peptide; (lane *2*) immunoprecipitation from *p21+/-* $p57^{+/-m}$ muscle extracts; (lane *3*) immunopricipitation from $p21^{-/-}$ $p57^{+/-m}$ muscle extracts. (*J*) Quantitation of assays in *I* by PhosphorImager. Scale bars, 200 μm.

diaphragm of double mutants when compared to the wild type (Fig. 4C,D). In the body wall, wild-type embryos display three layers of skeletal muscle (Fig. 4E), each of which is diminished in the double mutants (Fig. 4F).

It is possible that the skeletal muscle defects observed in double mutants arise from defects in primary myogenesis by which myoblasts are specified and migrate out of somites to various places in the embryo to form skeletal muscles later during secondary myogenesis (Kelley 1983). At E13.5, a time when primary myogenesis is well under way, however, we observed similarly patterned skeletal muscle groups in the double mutant when compared to a wild-type embryo (Fig. 3I,J). In addition, no difference in the morphology of somites are detected between double mutants and wild-type animals at E9.5 (data not shown). Therefore, we conclude that the skeletal muscle defects in the double mutants are a result of problems in secondary myogenesis, similar to the defects observed in mice lacking myogenin (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995).

Absence of both p21 and p57 lead to overproliferation, endoreplication, and apoptosis

Given the biochemical function of p21 and p57 as CKIs, proliferation rates and Cdk2 kinase activities in skeletal muscle from animals with different genotypes were examined. BrdU pulse labeling in E16.5 embryos demonstrated a greater than twofold increase in the number of cells undergoing DNA synthesis in the intercostal muscle region of double mutants when compared to those of either $p21^{+/-}$ $p57^{+/+}$ or $p21^{+/-}$ $p57^{+/-m}$ animals (Fig. 5A–C). We have also noticed incorporation of BrdU in the nuclei of residual myotubes in double mutants (Fig. 5D, arrows), indicative of endoreplication. This is never observed in wild type or single mutants (data not shown). As a result, double mutants frequently display enlarged and unusually shaped nuclei in the residual myotubes (Fig. 5, cf. E and F). In agreement with the observed elevation in proliferation rates in the double mutants, a threefold higher Cdk2 activity toward its physiological substrate Rb protein was detected in the muscle extracts made from $p21^{-/-}$ $p57^{+/-m}$ relative to $p21^{+/-}$ $p57^{+/-m}$ animals (Fig. 5I,J), indicating that p21 and p57 are functioning as CKIs in vivo.

The fact that double mutant animals exhibit greatly reduced skeletal muscle mass appears to contradict the fact that they also display increased proliferation. This apparent inconsistency could be explained by an increase in cell death by apoptosis in double mutants. To test that hypothesis, TUNEL assays were performed on transverse sections of E16.5 embryos. Apoptotic cells were readily detected in the double mutants (Fig. 5H) but not in the wild type (Fig. 5G) or single mutants (not shown), explaining the apparent discrepancy. Together, these data indicate that in the absence of both p21 and p57, myoblasts cannot properly withdraw from the cell cycle in response to differentiation signals, leading to overproliferation, endoreplication, and apoptosis.

The block to differentiation in $p21^{-/-}$ $p57^{-m/+}$ muscle is after the myogenin expression step

The skeletal muscle and rib phenotypes of $p21^{-/-}$ $p57^{-m/+}$ double mutants are nearly identical to those of mice lacking myogenin. This coincident phenotype could be explained if $p21^{-/-}$ $p57^{-m/+}$ animals failed to make myogenin or if myogenin null animals failed to express p21 and p57. To address this, we examined myogenin expression in $p21^{-/-}$ $p57^{-m/+}$ double mutants. In situ hybridization with a *myogenin* antisense probe revealed equivalent expression of *myogenin* mRNA in all skeletal muscles examined in both wild-type and $p21^{-/-}$ $p57^{-m/+}$ double mutant animals (Fig. 6A,B; data not shown). Western blot analysis of hind limb muscle extracts using a monoclonal antibody against myogenin also demonstrated similar levels of protein expression and electrophoretic mobility of myogenin proteins among littermates with various genotypes (Fig. 6C). To test the functionality of the myogenin protein, we examined transcription of a gene thought to be downstream of *myogenin*, *MEF2C*, a MADS box-containing transcription factor involved in myogenesis. MEF2C is expressed at lower levels in double mutants than that in the wild-type control (Fig. 6D,E). It should be noted that we cannot distinguish between reduced MEF2C expression versus selective loss of MEF2C-expressing cells through apoptosis producing the appearance of reduced MEF2C expression. Nevertheless, these data together demonstrate that the skeletal muscle phenotypes observed in

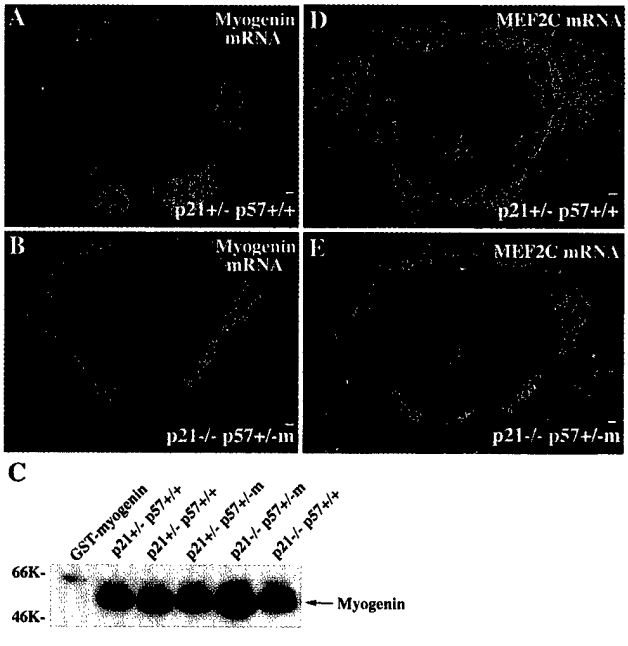

Figure 6. Expression of skeletal muscle differentiation factors in $p21^{-/-}$ $p57^{-m/+}$ double mutants. (*A,B*) *myogenin* expression detected with in situ hybridization on transverse sections of E18.5 embryos. (*C*) A Western blot of hind limb muscle extracts probed with a monoclonal antibody against myogenin. (*D,E*) MEF2C expression detected by in situ hybridization on transverse sections of E18.5 embryos.

$p21^{-/-}$ $p57^{-m/+}$ double mutants are not due to impaired expression of myogenin, a major skeletal muscle differentiation transcription factor but may result from an inability of myogenin or a myogenin-controlled factor to properly function in the absence of proper cell-cycle exit (see Discussion).

p21 and p57 expression are parallel to myogenin expression in the myogenic pathway

The normal expression of myogenin in the $p21^{-/-}$ $p57^{-m/+}$ animals indicates that these two CKIs are not upstream of *myogenin* in the myogenic pathway. It is possible that the opposite is true, however—that *myogenin* actually controls the transcription of both *p21* and *p57*. To test that possibility, we investigated the ability of myogenin to induce p21 and p57 expression in myogenin-expressing 10T1/2 fibroblasts. Unlike MyoD-programmed 10T1/2 cells induced to differentiate, myogenin was incapable of inducing p21 expression upon serum withdrawal although it could induce its own transcription and cause the formation of myotubes (Fig. 7D). We were unable to detect induction of p57 in either MyoD- or myogenin-programmed cells in vitro, suggesting that p57 may be controlled by a novel signal transduction pathway. To determine the dependency of inhibitor expression on the presence of myogenin in vivo, we examined *p57* and *p21* expression in *myogenin* null animals by in situ hybridization. As shown in Figure 7, A and B, p57 is expressed at equivalent levels in *myogenin* null mice compared to that of wild-type controls. Taken together with our previous report showing normal *p21* expression in *myoD/myogenin* double mutant animals (Parker et al. 1995), we conclude that *myogenin* is not required for either *p21* or *p57* expression. Therefore, myogenin is neither necessary nor sufficient for the expression of the two inhibitors, resulting in the placement of *p21* and *p57* in parallel to *myogenin* in the myogenic pathway (see Fig. 7E,F).

p21 and p57 are coexpressed in the same cells

The redundancy observed between p21 and p57 can be explained by redundant activities within each individual myocyte. Alternatively, p21 and p57 can each be required individually in different cell types representing distinct but redundant myogenic lineages. Such lineages have been hypothesized to explain the apparent redundancy between MyoD and Myf5. Furthermore, previous analysis of p57 expression in the nuclei within myotubes revealed that only half of the nuclei contained p57 protein, consistent with a two lineage hypothesis (Zhang et al. 1997). To explore this we sought to determine whether p57 and p21 showed colocalization. For technical reasons we were unable to visualize p21 protein in myotubes harvested from mice. To circumvent this, we harvested myoblasts from mice, differentiated them into myotubes in vitro, and analyzed p21 and p57 protein by indirect immunofluorescence. Under these circumstance p21 and p57 were found to be completely colocalized in each nucleus in the myotubes formed (Fig. 7C). This indicates that the redundancy between p21 and p57 is within an individual cell and not between cells. The difference in the number of nuclei expressing p57 in vitro versus in vivo is probably due to the fact that these cells in vitro are synchronized in their differentiation process. After differentiation, p57 levels drop, and it is

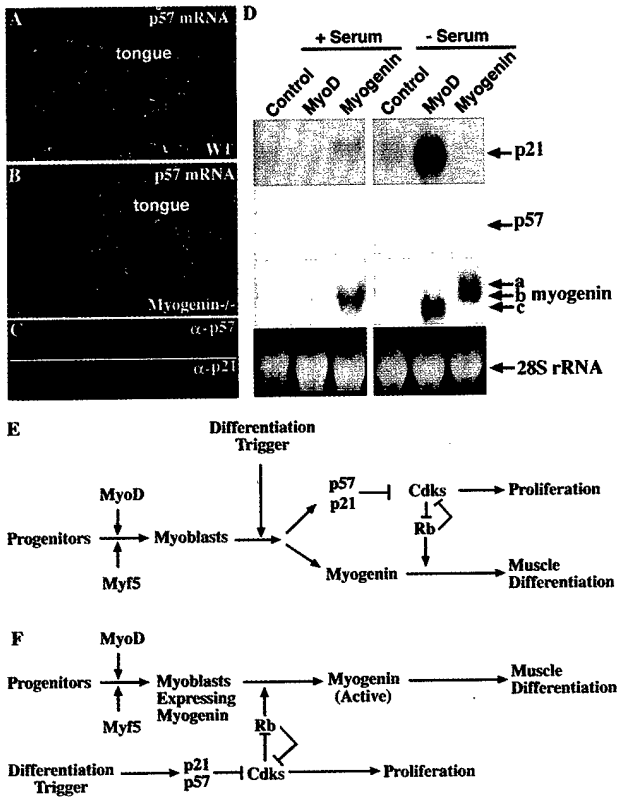

Figure 7. Myogenin is neither required nor sufficient for the expression of p21 and p57. (*A,B*) p57 expression detected with in situ hybridization on coronal sections of E15.5 embryos. (*C*) Colocalization of p21 and p57. Myotubes formed in vitro from primary embryonic myoblasts were stained with rabbit anti-p57 and goat anti-p21 polyclonal antibodies. p57 was visualized with a FITC-conjugated secondary antibody. p21 was visualized with a biotin-conjugated secondary antibody followed by Texas red-linked streptavidin. (*D*) A Northern blot of total RNA isolated from proliferating (+ serum) and differentiated (- serum) 10T1/2, MyoD-10T1/2, and myogenin–10T1/2 cells was probed sequentially with *p21*, *p57*, and *myogenin*. EtBr-stained 28S rRNA was used as a loading control. Three different sizes of *myogenin* mRNA are observed; *a* corresponds to the endogenous *myogenin* mRNA induced by myogenin, *b* corresponds to the *myogenin* transgene, and *c* corresponds to the endogenous myogenin mRNA induced by MyoD. The endogenous *myogenin* transcripts induced by MyoD and myogenin are of a different size. (*E*) A model for myogenesis in which *myogenin*, *p21*, and *p57* are coordinately induced by a differentiation triggering signal to coordinate muscle cell differentiation. (*F*) A different model for myogenesis in which induction of *p21* and *p57* is the critical event that triggers muscle cell differentiation (see text for details).

possible that the absence of p57 observed in 50% of nuclei in vivo reflects nuclei that have already reduced p57 expression.

Discussion

Proliferation control is vital to a developing organism. CKIs are good candidates for molecules providing the controls on cellular proliferation required for embryonic developmental programs because of their biochemical properties and their patterns of expression. For example, p21 was found to be highly expressed in a number of terminally differentiating tissues (Parker et al. 1995). Surprisingly, however, mice lacking p16, p21, or p27 display normal embryonic development (Deng et al. 1995; Serrano et al. 1996; Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996), suggesting that other cell-cycle regulatory mechanisms might exist to compensate for their loss. The present work reveals such a redundancy and demonstrates that two CKIs, p21 and p57, cooperate to control proliferation and differentiation in multiple tissues in mice.

p21 and p57 control differentiation and morphogenesis but not the cell cycle in the lung

The lung develops through interactions between an epithelial tissue that lines airways and a mesenchymal tissue that surrounds the epithelium. It is likely that in the absence of p21 and p57, epithelium–mesenchyme interactions are somehow disrupted in the formation of primitive alveoli. This disruption is stage specific, as development of the bronchial tree is unaffected. Thus far, we have been unable to define more precisely the cell types defective in the lung of mutant animals because p21 and p57 are coexpressed in a number of different cell types and no morphological differences are observed among them.

The lung defects appear to be quite distinct from those observed in skeletal muscle. First, MyoD and myogenin do not have a role in lung development, so novel signal transduction pathways are likely to control p21 and p57 in lung differentiation pathways. Second, in the absence of p57, muscle can properly differentiate with a single copy of the *p21* gene, whereas the lung shows haploinsufficiency under these conditions. This is likely to reflect differences in the other differentiation information present in these cell types. Third, unlike skeletal muscle, the defects in the formation of primitive alveoli do not appear to be a result of overproliferation or apoptosis. It is possible that these two CKIs contribute directly to the differentiation of either epithelial or mesenchymal cells in a cell-cycle-independent way. Alternatively, increased Cdk activity caused by the lack of CKIs might be insufficient to drive the G_1/S transition but sufficient to interfere with the differentiation processes. Analysis of *Rb* mutants has shown that it is possible to separate the cell cycle and differentiation functions of Rb (Sellers et al. 1998), although whether it is possible to separate these functions by differential Cdk-dependent phosphorylation is not known. Alternatively, there could exist Cdk-dependent pathways parallel to Rb that are required for differentiation. In support of this, Skapek et al. (1996) have shown that additional cyclin D1 could block myogenesis even in the presence of nonphosphorylatable and presumably constitutively active Rb. Clearly, however, different tissues can be dependent on the same two regulators in different ways, underscoring the complex relationship between the cell cycle and development.

p21 and p57 are required for skeletal muscle differentiation

Several studies have suggested a coupling between cell-cycle exit and differentiation of myoblasts (Bischoff and Holtzer 1969; Nadal-Ginard 1978; Clegg et al. 1987). Furthermore, a large body of evidence indicates that Rb is necessary for the permanent withdrawal of skeletal muscle cells from the cell cycle (Gu et al. 1993; Schneider et al. 1994; Novitch et al. 1996; Mulligan and Jacks 1998). Because Rb is a Cdk substrate and is inactivated by Cdk phosphorylation, Cdk regulation is implicated as a key event in muscle development. How Cdks are regulated to initiate muscle differentiation has been a difficult issue to resolve because in vitro differentiation systems utilize nonphysiological stimuli such as serum deprivation to initiate muscle cell differentiation. Thus, the mechanistic details of the initial cell-cycle exit have remained unexplored. This work identifies an important part of the mechanism through which muscle cells exit the cell cycle and initiate differentiation in vivo. Mice lacking both p21 and p57 display severe skeletal muscle defects, manifested as a failure to form myotubes, increased proliferation and apoptosis rates of myoblasts, and endoreplication in the nuclei of residual myotubes. The skeletal muscle phenotypes are similar to those observed in $Rb^{-/-}$ mice rescued to term by a Rb transgene expressed at low levels (Zacksenhaus et al. 1996). This strongly suggests that the primary role of p21 and p57 is to down-regulate Cdk activity and maintain Rb in an active (hypophosphorylated) form. Inactivation of both p21 and p57 leads to higher Cdk2 kinase activity toward Rb in the skeletal muscle (Fig. 5I,J). Other CKIs have also been implicated in the regulation of pocket proteins by virtue of phenotypic similarities of mutant mice; p27 and p57 have been implicated in Rb control in the pituitary and lens, respectively (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996; Zhang et al. 1997), and p57 has been implicated in p107/130 regulation in chondrocyte differentiation (Cobrinik et al. 1996; Yan et al. 1997; Zhang et al. 1997).

p21, p57, and Rb function in muscle development at the myogenin step

To fully differentiate, cells must not only cease proliferation but also initiate a differentiation program of cell

type-specific transcription. An increasing body of evidence suggests that in addition to its role in cell-cycle exit, hypophosphorylated Rb directly promotes differentiation by acting as a transcriptional coactivator of differentiation transcription factors. During adipocyte differentiation, an interaction between Rb and members of the C/EBP family of transcription factors occurs that potentiates C/EBP DNA binding and transcriptional activity (Chen et al. 1996). In terminally differentiating keratinocytes, Rb interacts with c-Jun and stimulates its transcriptional activity (Nead et al. 1998).

In vitro studies coupled with our in vivo genetic studies suggest that Rb may carry out a similar function in muscle cell differentiation. The skeletal muscle differentiation pathway is controlled by a group of bHLH myogenic transcription factors that form a transcriptional hierarchy in which MyoD and Myf5 are redundant and act upstream of myogenin, and myogenin is the major differentiation factor (for review, see Olson and Klein 1994). Gu et al. (1993) have shown that Rb interacts physically with myogenic transcription factors such as MyoD and myogenin. Furthermore, transcriptional activation of a skeletal muscle-specific promoter by MyoD requires the presence of a functional Rb protein (Novitch et al. 1996). If Rb were required for the function of a particular muscle transcription factor, one might expect the phenotype of *Rb*-deficient animals and $p21^{-/-}$ $p57^{-m/+}$ animals to resemble the phenotype of animals lacking that transcription factor. MyoD mutants have no phenotype unless combined with Myf5, in which case they are completely defective in formation of any muscle precursor cell types. This is clearly different from the $Rb^{-/-}$ and $p21^{-/-}$ $p57^{-m/+}$ phenotypes. However, skeletal muscle defects in $p21^{-/-}$ $p57^{-m/+}$ double mutants and $Rb^{-/-}$ mutants rescued by a hypomorphic Rb transgene bear strong similarity to the defects in *myogenin* null mice. This could be due to the failure of a part of the MyoD/Myf5 program, for example, the failure to express myogenin. However, myogenin is expressed normally in the double mutants, suggesting that myogenin, and not MyoD or Myf5, fails to function in the absence of proper Cdk regulation. Furthermore, expression of MEF2C, a myogenic transcription factor thought to be downstream of myogenin (Cserjesi et al. 1994; Edmondson et al. 1994; Molkentin et al. 1995), is reduced in $p21^{-/-}p57^{-m/+}$ animals, consistent with a defect in myogenin activity or an earlier step. The involvement of myogenin in the Rb-dependent step is supported by Tedesco et al. (1995), who have shown that SV40 T antigen blocks myogenesis after myogenin has been expressed, and Novitch et al. (1996), who demonstrated that in the absence of Rb, MyoD induces the expression of both p21 and myogenin, but not later differentiation markers such as MHC, which depend on the function of myogenin (Hasty et al. 1993; Nabeshima et al. 1993; Venuti et al. 1995). Whereas myogenin has been shown to interact physically with Rb in vitro, our studies do not address whether this connection is direct. The failure to inactivate Cdks could block the function of *myogenin*, a parallel gene required together with *myogenin*, or a gene directly downstream of myogenin, and any one of these could physically require hypophosphorylated Rb.

Muscle differentiation in vivo—what pulls the trigger?

A key issue in muscle development is what triggers the differentiation process. MyoD and Myf5 have been shown to specify the myoblast lineage. Once specified, myoblasts continue to proliferate until they receive a differentiation signal that has not yet been identified. The process initially affected by this signal is not known. However, in vitro cell culture experiments have demonstrated that serum deprivation can trigger differentiation and therefore substitute for the in vivo signal. The dependency on Rb for this process, coupled with the in vivo dependency on p21 and p57, suggests that the relevant function of serum deprivation is cell-cycle arrest through Cdk inactivation. p21 and p57 are essential targets of signal transduction pathways intended to control muscle differentiation in vivo. A critical question is whether p21 and p57 up-regulation in vivo is the trigger for the differentiation cascade or plays a critical downstream coordinating function, or both.

Combining our knowledge of the regulation of myogenic differentiation, together with the transcriptional regulation of *p21* and *p57*, sheds some light on these issues. First, both *p21* and *p57* are expressed normally in mice deficient for either MyoD or Myf5 (Parker et al. 1995; P. Zhang and S. Elledge, unpubl.), although they may require one of these. Second, MyoD in 10T1/2 cells or C2C12 cells can direct the expression of *p21* but, intriguingly, not *p57* (Fig. 7; data not shown), suggesting that *p57* is under the control of a novel signaling pathway that is not active in the in vitro model systems, although it is possible that expression of *p57* is inactivated in established cell lines in a manner that does not reflect its true in vivo regulation. Finally, it is clear that myogenin is neither necessary nor sufficient for the expression of p21 or p57 and vice versa, indicating that all three are expressed in parallel. In vitro model systems such as C2C12 cells show that myogenin expression is low initially and then highly induced in response to serum deprivation (Parker et al. 1995; Andres and Walsh 1996). If myogenin expression in vivo is activated after the differentiation trigger is pulled, the fact that myogenin is fully expressed in the absence of p21 and p57 indicates that these CKIs cannot be the event initially triggering myocyte differentiation. The major caveat in this analysis is whether or not myogenin expression in vivo follows the in vitro model or whether in vivo myogenin is expressed in an inactive form prior to the triggering event (see below). Alternatively, it is possible that both p21 and p57 are part of a forward feeding differentiation switch that once initiated, perhaps by transient cell-cycle exit, maintains the differentiation process, similar to the models put forth for MyoD and p21 (Halevy et al. 1995; Parker et al. 1995).

On the basis of these observations and information from in vitro muscle differentiation systems, we propose a myogenesis model as depicted in Figure 7E. MyoD and

Myf5 specify cells to adopt the myoblast fate. Myoblasts then migrate and proliferate. In response to differentiation signals of unknown origin, expression of myogenin, the driving force in myoblast differentiation, is activated. At the same time, both p21 and p57 expression are induced and act to inhibit Cdk activity, causing G_1 arrest and maintaining Rb in its hypophosphorylated and active form. Rb then works in conjunction with myogenin (or an unknown factor with a similar function) to activate a program of muscle-specific gene expression that executes the differentiation process. A candidate Rb-responsive alternative to myogenin might be MEF2C, a myogenic transcription factor thought to be downstream of myogenin. Although MEF2C mutant mice are embryonic lethal and the role of MEF2C in muscle development has not been assessed, it is likely to be required for muscle cell differentiation because its *Drosophila* homolog, Mef2, is required for muscle formation (Bour et al. 1995; Lilly et al. 1995). Mef2 is the only *Drosophila* myogenic homolog required for muscle development. In this model, CKIs could have additional nonessential roles besides activation of the myogenin-dependent step, such as enhancing MyoD function or non-cell-cycle roles in differentiation that are not indicated in the model. Both p21 and p57 have domains in addition to the Cdk inhibitory domain that are likely to have other functions, possibly involved in differentiation.

A second possible model for myogenesis is shown in Figure 7F in which p21 and p57 induction acts as the trigger for muscle differentiation. In this model, after specification, myoblasts express myogenin in an inactive form. The differentiation trigger functions by activating p21 and p57 expression, which inhibits Cdk activity causing G_1 arrest and Rb activation. Rb then functions together with myogenin to carry out differentiation. We favor the first model because it is consistent with the results from in vitro muscle differentiation systems. However, the second model makes two testable predictions. If true, myogenin will be expressed in proliferating myoblasts and will be expressed prior to p21 and p57 induction. Furthermore, ectopic activation of p21 or p57 expression in myoblasts should induce muscle differentiation.

Regardless of whether p21 and p57 are the initiating molecules for muscle development, the profound defects observed in p21/p57 double mutant mice demonstrate the pivotal importance of coordination between proliferation and differentiation in development and demonstrate that multiple layers of Cdk regulation have been selected during evolution to ensure this coordination.

Materials and methods

Genotyping animals

p21-deficient mice were kindly provided by Philip Leder (Harvard Medical School, Boston, MA). We have developed PCR protocols to identify wild-type and disrupted alleles of p21 and p57, using a set of four primers for p21 and three primers for p57. For p21, the sequences are primer 1, TCCTGGTGATGTCCGACCTG; primer 2, TCCGTTTTCGGCCCTGAG; primer 3, GCGAGGATCTCGTCGTGAC, and primer 4, TCATCAATTTATGCAGAC. For p57, the sequences are primer 1, CGTCCACAGGCCGAGTGC; primer 2, GCTGCGGAGGTACACGTCG; and primer 3, GCGAGGATCTCGTCGTGAC. Detailed protocols are available on request.

Gross and histological analysis

Embryos or placentas were fixed in 4% paraffin aldehyde dissolved in PBS for several hours to overnight at 4°C, depending on the size of the specimen. Fixed samples were dehydrated using ascending concentrations of ethanol, cleared in xylene, and embedded in paraffin wax. Embedded samples were sectioned at 3 μm. For histopathological evaluation, tissue sections were stained with H&E. Skeleton staining was performed as described (Ramirez-Solis et al. 1993)

Immunofluorescence analysis

MHC was detected using a monoclonal antibody (Sigma, clone MY-32) and visualized with Texas Red-conjugated secondary antibody (Amersham). p57 was stained as described (Zhang et al. 1997). For cell proliferation assays, pregnant mice were injected with BrdU (0.1 μg/gram body weight) 2 hr prior to delivery by cesarean section, and positive cells identified with an anti-BrdU mAb (Dako) and visualized with FITC-conjugated secondary antibody (Amersham). Apoptotic cells were detected with a kit from Trevegene, and the assay was performed as recommended by the manufacturer.

In situ hybridization

In situ hybridization analysis was performed as described (Parker et al. 1995). Sense and antisense probes were generated from linearized plasmid templates obtained from the following sources: myogenin, Eric Olson (University of Texas Southwestern Medical Center, Dallas); p21 and p57 (our laboratory); MEF2C, CC10, SP-A, SP-B, and SP-C EST clones from Genome Systems (St. Louis, MO).

Culture of primary myoblasts

Hind limb muscles dissected from E18.5 embryos were digested with 0.5 ml of dispase (Boehringer Mannheim, Indianapolis, IN.) at 37°C for 30 min, and tissues were dislodged by repeated pipetting. The resulting cell suspensions were mixed with DMEM (GIBCO) containing 20% FBS and appropriate antibiotics, and incubated in 10-cm tissue culture dishes for 30 min at 37°C to adhere fibroblasts onto the dish. The supernatants containing myoblasts (and fibroblasts) were plated in appropriate tissue culture vessels. After a 48-hr recovery period, myoblasts were induced to differentiate with DMEM containing 2% horse serum. Myotubes appear by 48 hr after induction. For immunofluorescence staining, cells were fixed in cold methanol (−20°C).

Protein and mRNA analysis

Hind limb skeletal muscle tissues dissected from E19 embryos were lysed in NP-40 buffer (Harper et al. 1993) and cleared by centrifugation. Thirty micrograms of protein was immunoblotted with a monoclonal anti-myogenin antibody available from Hybridoma Bank (University of Iowa, Iowa City) using ECL detection (Amersham). GST–myogenin was purchased from Santa

Cruz Biotechnology (Santa Cruz, CA). mRNA expression was analyzed by Northern blotting total RNA isolated from 10T1/2 fibroblasts ectopically expressing MyoD or myogenin (Parker et al. 1995). Cdk2 kinase was immunoprecipitated from muscle extracts using a polyclonal antibody against Cdk2 (Santa Cruz, CA) and its activity assayed using *Escherichia coli* expressed Rb protein (kindly provided by David Goodrich, University of Texas M.D. Anderson Cancer Center, Houston) as substrates.

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Cooperation between the *Cdk* inhibitors $p27^{KIP1}$ and $p57^{KIP2}$ in the control of tissue growth and development

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RESEARCH COMMUNICATION

Cooperation between the *Cdk* inhibitors $p27^{KIP1}$ and $p57^{KIP2}$ in the control of tissue growth and development

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Cell cycle exit is required for terminal differentiation of many cell types. The retinoblastoma protein *Rb* has been implicated both in cell cycle exit and differentiation in several tissues. *Rb* is negatively regulated by cyclin-dependent kinases (*Cdk*s). The main effectors that down-regulate *Cdk* activity to activate *Rb* are not known in the lens or other tissues. In this study, using multiple mutant mice, we show that the *Cdk* inhibitors $p27^{KIP1}$ and $p57^{KIP2}$ function redundantly to control cell cycle exit and differentiation of lens fiber cells and placental trophoblasts. These studies demonstrate that $p27^{KIP1}$ and $p57^{KIP2}$ are critical terminal effectors of signal transduction pathways that control cell differentiation.

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Proper development of an organism requires an integration of cell cycle exit and differentiation pathways. Proliferation is positively regulated by cyclin-dependent kinases (*Cdk*s), a family of highly regulated enzymes that link proliferative signals with mechanical aspects of cell duplication. Acting in opposition to *Cdk*s are *Cdk* inhibitors, *CKI*s. Two families of *CKI*s have been identified. The $p21^{CIP1}$ family contains p21, $p27^{KIP1}$, and $p57^{KIP2}$ and inhibits all kinases involved in the G_1/S transition, whereas the $p16^{INK4a}$ family, including *p15*, *p16*, *p18*, *p19*, inhibits *Cdk4* and *Cdk6* specifically (for review, see Harper and Elledge 1996). The biochemical activities and patterns of expression of *CKI*s during development (Matsuoka et al. 1995; Parker et al. 1995), together with data derived from in vitro differentiation systems (Guo et al. 1995; Halevy et al. 1995; Parker et al. 1995), implicate these proteins as the primary effectors of signaling pathways that control cell cycle exit, an event that is critical for differentiation. However, of all the *CKI*s, only *p57* is required for embryonic development (Deng et al. 1995; Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996; Serrano et al. 1996; Zhang et al. 1997; Yan et al. 1997). Loss of *p57* results in proliferative disorders in the lens and in cartilage, and defects in development of several tissues (Yan et al. 1997; Zhang et al. 1997).

Insights into the question of how cell cycle arrest and differentiation are integrated have come from the analysis of embryonic lens development. The lens is composed of differentiated lens fiber cells capped on the anterior surface by a layer of immature, mitotic epithelial cells (McAvoy 1980; Piatigorsky 1981). Formation of this structure involves spatially controlled proliferation and differentiation events that are dependent on the retinoblastoma (*Rb*) gene product, a critical target of *Cdk*s. Loss of *Rb* leads to defects in cell cycle arrest and differentiation, as well as increased p53-dependent apoptosis (Morgenbesser et al. 1994; Liegeois et al. 1996). In other differentiation systems such as skeletal muscle, *Rb* appears to play a dual role; it acts as a growth suppressor facilitating G_1 arrest and is also required for activating the transcriptional program that brings about differentiation (for review, see Mulligan and Jacks 1998), possibly through physical association with critical transcription factors (Gu et al. 1993; Chen et al. 1996; Nead et al. 1998). Although cell cycle arrest and activation of differentiation processes may involve separable functions of *Rb* (Sellers et al. 1998), available evidence suggests that both of these functions require inhibition of *Cdk*s (Rao et al. 1994; Skapek et al. 1995, 1996). However, the main effectors that down-regulate *Cdk* activity to activate *Rb* are not known in the lens or other tissues. In this study, using multiple mutant mice, we show that $p27^{KIP1}$ and $p57^{KIP2}$ function together in a redundant manner to control cell cycle exit and differentiation in the lens and the placenta.

Results and Discussion

Ocular lens development involves several steps. By embryonic day 11.5 (E11.5), a sphere of epithelial cells have formed the lens vesicle. At this stage, cells in the posterior region undergo cell cycle exit and begin to elongate toward the anterior wall. Three days later, elongation is complete and these differentiated fiber cells are capped on the anterior wall by a layer of immature epithelial cells. These cells proliferate and migrate to the equatorial zone where they exit the cell cycle and differentiate to form secondary lens fiber cells (for review, see McAvoy 1980; Piatigorsky 1981). Cells in the equatorial zone express high levels of *p57* (Fig. 1D; Zhang et al. 1997), and loss of *p57* allows these cells to continue to proliferate temporarily (Zhang et al. 1997). However, *p57*-deficient lens cells eventually undergo cell cycle exit and differentiate into lens fiber cells. These lenses are relatively normal but, in some genetic backgrounds, accumulate vacuoles indicative of incomplete lens fiber cell elongation and/or apotosis. The ability of *p57*-deficient

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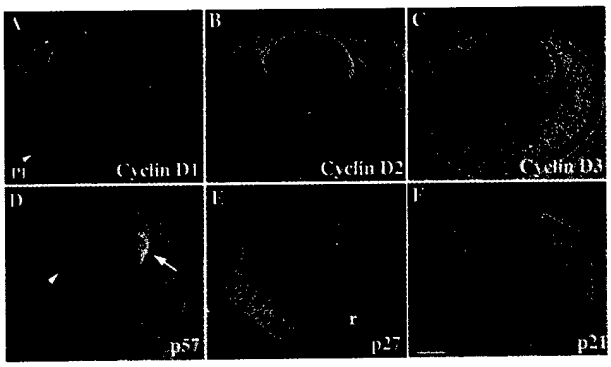

Figure 1. Expression of cell cycle regulatory genes during lens development. In situ hybridization was performed on transverse sections through the eye region of an E15.5 embryo using antisense probes as indicated. The arrow in *A* points to the pigmented epithelium (PE), which falsely stains positive for all probes because of the presence of pigmented granules in these cells. Arrows in *D* indicate cells in the equatorial zone of the lens that express high levels of *p57*. *p27* is expressed in the equatorial zone and in the retina (r). Scale bar, 200 μm.

lens cells to differentiate, albeit with reduced kinetics, implies the existence of a second regulatory pathway controlling cell cycle exit in this tissue.

Cell cycle exit could be achieved by down-regulating cyclins or by inducing additional *CKI*s. To examine these possibilities, we performed in situ hybridization analysis to determine the transcriptional status of *CKI*s and D-type cyclins in the lens during differentiation. At day E15.5, all three D-type cyclin mRNAs are expressed in the lens, with *D2* showing the strongest expression (Fig. 1A–C). *D2* and *D3* also show mRNA expression in the posterior chamber, which contains primarily differentiated cells. *D1* and *D2* had been shown previously to be expressed at day E13.5 in cells of the anterior epithelia and equatorial zone, and *D2* expression was observed in the posterior chamber (Fromm and Overbeek 1996), suggesting that additional inhibitory signals are likely to be required to counteract their growth-promoting activities. Consistent with this notion, we observed expression of a second *CKI*, *p27*, in the same cells as *p57* in the equatorial zone (Fig. 1E) and in the posterior chamber of the lens. In contrast, *p21* transcripts were not detected in the lens (Fig. 1F).

Previous studies did not detect defects in lens development in *p27*-deficient mice (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996). To determine whether the coincident expression of *p27* and *p57* was indicative of a redundant function, we generated mice mutant for both of these *CKI*s. Male $p27^{-/-}p57^{+/+}$ and female $p27^{+/-}p57^{+/-p}$ (p denoting paternal origin of the mutant *p57* allele) mice were mated to enrich for double mutants. Because the $p57^{KIP2}$ gene is imprinted, only the allele inherited from the mother (m denoting maternal origin) need be mutant to produce phenotypically null offspring (Zhang et al. 1997). No *p57*-deficient animals survived to the time of genotyping (10 days of age), irrespective of the status of *p27*, confirming our earlier finding that *p57* is essential for neonatal survival (Zhang et al. 1997). Dead pups found in newborn litters were shown to have a $p57^{+/-m}$ genotype. Although expected frequencies of mice with all possible genotypes were found in embryos harvested from E13.5 to E18.5 (data not shown), we observed a significant incidence of embryonic lethality in *p57* mutant embryos, as has been reported (Yan et al. 1997; Zhang et al. 1997). Interestingly, deletion of *p27* significantly increased the frequency of embryonic lethality by a factor of 2 when the viability of $p27^{-/-}p57^{+/-m}$ animals (20% lethality) are compared to that of $p27^{+/-}p57^{+/-m}$ animals (10% lethality). However, we were unable to make a valid comparison between the lethality of $p27^{-/-}p57^{+/-m}$ (20% lethality) and $p57^{+/-m}$ animals from our original report (10% lethality; Zhang et al. 1997), because of different genetic backgrounds (see Materials and Methods). Embryos died over a wide window between day E12 and E16.5, and embryos that were alive at the time of harvesting showed heterogeneous degrees of growth retardation indicative of intermediate penetrance, likely resulting from the nature of mixed genetic backgrounds among these animals (see Materials and Methods). Histopathological examination of mutant embryos failed to show defects in the cardiovascular system or erythropoiesis, common sources of embryonic lethality. However, defects were observed in the placenta of mutant animals, an organ critical for fetal development and survival. This defect will be discussed below.

Embryos from these crosses that were not affected by placental defects were examined for developmental phenotypes, including those reported previously for the $p57^{+/-m}$ mutant. All affected tissues displayed phenotypes equivalent to those seen in *p57*-deficient embryos (Zhang et al. 1997), with the exception of the lens in which a profound defect was observed in the $p27^{-/-}p57^{+/-m}$ double mutants. It should be noted that phenotypically, $p27^{-/-}$ lenses were indistinguishable from wild-type lenses (data not shown) and $p27^{+/-}p57^{+/-m}$ lenses were indistiguishable from lenses derived from $p57^{+/-m}$ mice (Fig. 2G–I). However, $p57^{+/-m}$ lens defects in the genetic background resulting from a cross with $p27^{-/-}$ mice are slightly more severe than those we observed previously (Fig. 2G–I; Zhang et al. 1997).

Dramatic defects in lens development of double mutant mice are apparent as early as E13.5, a time at which posterior cells have normally already initiated elongation into primary lens fiber cells (Fig. 2D). Most striking is the finding that lens vesicles from double mutant mice are filled with nuclei as assessed histologically (Fig. 2F). Although cells adjacent to the posterior wall fail to elongate in the double mutant, this effect, albeit less dramatic, is also seen in this background in $p57^{+/-m}$ mice (Fig. 2E,F). By E15.5, the posterior zone nuclear density has increased further in double mutants, and no nuclei are detected in wild-type or $p27^{-/-}$ mutants, and far fewer nuclei are present in $p27^{+/-}p57^{+/-m}$ or $p57^{+/-m}$ lenses (Figs. 2A–C and 3A–C). The appearance of large numbers of nuclei in the lens fiber cell compartment is consistent with ectopic proliferation. To verify this sup-

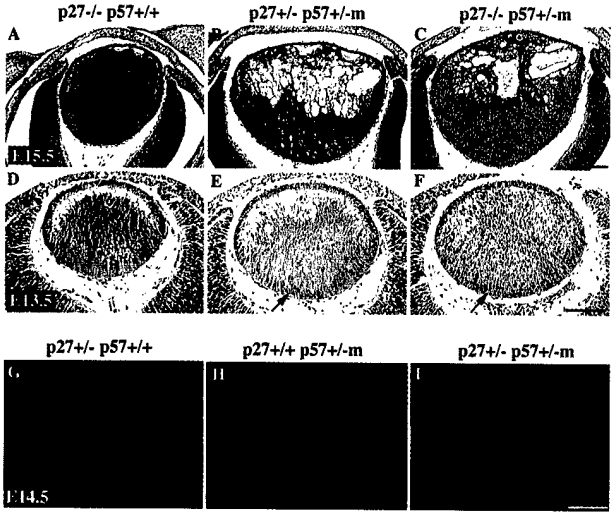

Figure 2. *p57* and *p27* are required for lens development. (*A–C*) Hematoxylin and eosin-stained sections of E15.5 lenses from the indicated genotypes. Nuclei at the posterior edge of $p57^{+/-m}$ (*E*) and $p27^{-/-}p57^{+/-m}$ (*F*) lenses are indicated by the arrow. (*G–I*) Nuclei in lens sections derived from E14.5 mice revealed by DAPI staining. Scale bar, 200 μm.

position directly, in situ BrdU incorporation assays were performed. The posterior chamber in E13.5 and E15.5 $p27^{-/-}p57^{+/-m}$ lenses contained many more actively dividing cells than did those of $p57^{+/-m}$ and $p27^{-/+}p57^{+/-m}$ heterozygous animals (Fig. 3D–F; data not shown). Sections from $p27^{-/-}p57^{+/-m}$ lenses at E15.5 displayed 66-fold more BrdU-positive cells than lenses from $p27^{+/-}p57^{+/+}$ mice and 8-fold more than lenses from $p27^{+/-}p57^{+/-m}$ mice, and similar values were observed at E13.5 (Fig. 3P). We also note that *p57* mutant lenses (regardless of *p27* status) are 15%–20% larger than wild-type lenses, and cataracts were apparent (Fig. 2A–C; data not shown).

The appearance of large vacuoles in the anterior chamber of *p57* mutant lens (irrespective of *p27* status) could be a result of the failure of fiber cells to elongate, or a consequence of cell death by both apoptosis and necrosis, or both. Lens fiber cell elongation is a hallmark of differentiation and requires proper temporal and spatial expression of lens crystallin proteins, expression patterns that serve as reliable markers of the lens differentiation program. Mice deficient for the transcription factor SOX1 display defects in the differentiation of lens fiber cells as indicated by the absence of induction of γ-crystallins, leading to incomplete elongation and large vacuoles in the lens (Nishiguchi et al. 1998). $p57^{+/-m}$ lenses display substantially reduced levels of β- and γ-crystallins (Fig. 3H,K), compared to $p27^{-/-}$ lenses (Fig. 3G,J), and crystallin expression is reduced to undetectable levels when combined with *p27* deficiency (Fig. 3I,L). Control experiments (not shown) indicate that the staining seen in the region of the vacuoles in the anterior of the chamber is due to nonspecific interactions (edge effect) of the secondary antibody used and does not reflect crystallin expression. These data indicate that *p57* and *p27* are required for proper lens fiber cell differentiation and elongation. To examine cell death, TUNEL assays were performed on E15.5 lenses. Apoptotic cells were detected in the posterior chamber of $p27^{+/-}p57^{+/-m}$ and $p27^{-/-}p57^{+/-m}$ mutant lenses, typically one apoptotic cell per 0.2-mm^2 cross section, but no apoptotic cells were detected in $p27^{-/-}$ lenses in this region (Fig. 3M–O). Because there are ~100 cross sections per lens, there are ~100 apoptotic cells per lens compared to zero in a *p27* null lens. Histological evidence of necrosis was also found and was most pronounced in regions immediately adjacent to vacuoles (data not shown). Thus, cell

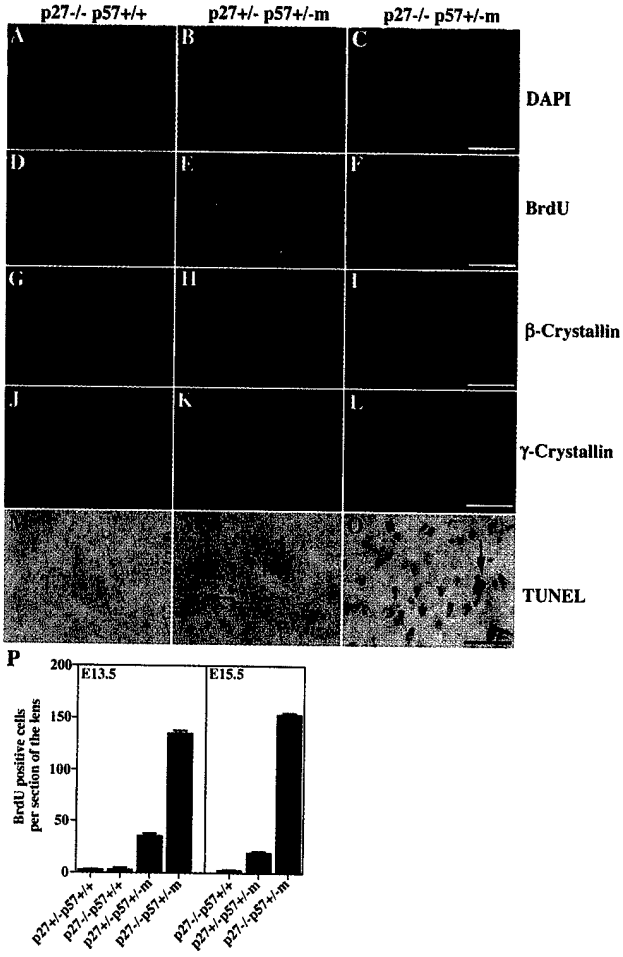

Figure 3. Overproliferation of lens fiber cells in *p27/p57* double mutants leads to compromised differentiation and increased apoptosis. (*A–C*) Nuclei of lens sections derived from E15.5 embryos revealed by DAPI staining. (*D–F*) BrdU incorporation assays demonstrate defects in cell cycle exit in $p27^{-/-}p57^{+/-m}$ lenses. (*G–L*) Immunofluorescence staining of β- and γ-crystallins demonstrates reduced expression of these two differentiation markers in the lens of $p57^{+/-m}$ mice (*H,K*) and the absence of expression in the lens of $p27^{-/-}p57^{+/-m}$ mice (*I, L*). (*M–O*) TUNEL assays detect apoptotic cells (arrows) in both $p57^{+/-m}$ and $p27^{-/-}p57^{+/-m}$ lenses. (*P*) Quantitation of BrdU incorporation assays at E13.5 and E15.5. BrdU-positive nuclei from a total of six sections for each genotype were determined, and the average is shown in the histogram along with the S.D.. Scale bars in *C, F, I,* and *L,* 200 μm; *O,* 50 μm.

death may also contribute to vacuoles in *p57*-deficient lenses.

Developmental defects in the placenta were also observed in $p27^{-/-}p57^{+/-m}$ mice. Of the several types of placentas, mice primarily have hemochorial placenta where maternal blood is no longer contained in blood vessels but is in direct contact with fetal trophoblasts that also embed fetal capillaries in the labyrinth zone. In placentas derived from $p57^{+/-m}$ single or $p27^{-/-}p57^{+/-m}$ double mutants, the labyrinth zone was less vascularized and contained more trophoblasts than those from wild-type or $p27^{-/-}$ mice (Fig. 4A, cf. a and b). The diameter of most mutant fetal capillaries was reduced to the size of a single fetal red blood cell, leading to the appearance of less vascularization. Normal placentas contain numerous open spaces (the fetal capillary and maternal blood sinus) that are replaced with trophoblasts in the mutant. We have found that this phenotype varies considerably, ranging from very little vascularization (Fig. 4A,b) to almost normal in those animals who survived to term (not shown), consistent with the variability in timing and rates of embryonic lethality. The degree of placental impairment correlates with size of the embryo, with more developmentally defective placentas containing smaller embryos.

In addition to reduced vascularization, placentas from *p57* mutant mice, regardless of the status of *p27*, contain areas that are marked by hyaline membranes in the labyrinth zone (Fig. 4A,c; data not shown). Necrosis was observed in these areas and is likely to be due to blockade of the blood supply by hyaline membranes. Hyaline membranes are formed in response to endothelium damage, as has been observed in the respiratory distress syndrome caused by capillary or alveolar epithelium damage (Kobizk and Schoen 1994). Given the biochemical functions of *p27* and *p57* and their roles in other tissues, we suspected that the absence of these *CKI*s might alter the differentiation of trophoblasts in the labyrinth zone, allowing them to proliferate inappropriately. This would result in limited space available for the fetal capillaries and maternal blood sinus, possibly leading to blood vessel damage and the formation of hyaline membranes. BrdU incorporation assays demonstrated increased proliferation in $p27^{-/-}p57^{+/-m}$ mutant placentas (Fig. 4C, cf. a and b). These assays were performed on a litter harvested at E18.5 when the normal placenta had already ceased proliferation to observe more easily the proliferation defects due to inhibitor loss. The fraction of BrdU-positive cells was greatly increased in placentas from $p27^{-/-}p57^{+/-m}$ mice relative to $p27^{+/-}$ and $p27^{-/-}$ placentas (25- and 10-fold, respectively) and was significantly larger (4-fold) than that found with $p27^{+/-}p57^{+/-m}$ placentas (Fig. 4C). There was considerable heterogeneity in the placental phenotypes of mutant animals. As shown in Table 1, a significant percentage of the $p27^{-/-}p57^{+/-m}$ embryos escape embryonic lethality, and placentas from these animals appear to be much less defective than those shown here. Furthermore, although the additional loss of *p27* increases proliferation rates, it does not significantly exacerbate the histological defects observed in *p57* mutant placentas. It does, however, change the penetrance of the placental failure phenotype, making the placenta twice as likely to fail. *p57* is highly expressed in the labyrinth zone but not the adjacent spongiotrophoblast zone (Fig. 4B,a). All of the placental defects are observed exclusively in the labyrinth zone, whereas other aspects of the placentas from these mutant mice are normal (not shown). *p27* is expressed both in labyrinth and spongiotrophoblast zone (Fig. 4B,c). This concordance of expression suggests that the defects observed are cell autonomous and indicates that *p27* can provide some compensatory function in the labyrinth zone in the absence of *p57*. Thus, both *p27* and *p57* are expressed in the tissue found defective in mutant embryos, suggesting that the phenotype is very likely to be cell autonomous.

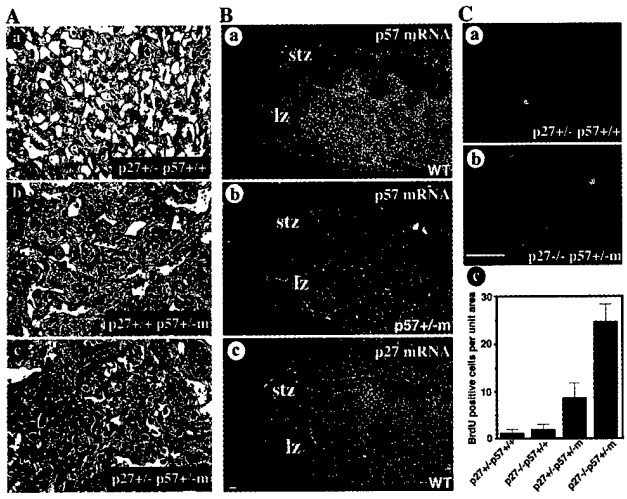

Figure 4. *p57* and *p27* are required for the proper development of the mouse hemochorial placenta. (*A*) Hematoxylin and eosin-stained E12.5 placenta sections from $p27^{+/-}$ (*a*), $p57^{+/-m}$ (*b*), and $p27^{+/-}p57^{+/-m}$ (*c*) mice. Arrows in *c* indicate hyaline membranes. (*B*) Expression of *p57* (*a*) and *p27* (*c*) in E12.5 placenta as detected by in situ hybridization. The specificity of the *p57* probe is demonstrated through the absence of signal in a placental section from a $p57^{+/-m}$ mouse (*b*). (*C*) BrdU incorporation assays reveal overproliferation in the labyrinth zone of $p27^{-/-}p57^{+/-m}$ placentas, as compared with $p27^{+/-}$, $p27^{-/-}$, or $p27^{+/-}p57^{+/-m}$ placentas. Placentas were harvested at E18.5. Quantitation of BrdU assays on embryos collected from a single litter are presented in *c*. Error bars represent the S.D. (lz) Labyrinth zone; stz, spongiotrophoblasts zone. Scale bars, 200 μm.

Analysis of mice lacking both *p27* and *p57* has revealed that these two *CKI*s cooperate to control cell cycle exit and differentiation in both the lens and placenta. In the lens, *p57* plays a role in cell cycle arrest in both posterior lens vesicle cells during primary differentiation and equatorial cells during secondary differentiation. *p57* levels increase dramatically in equatorial cells at the time of cell cycle exit and these cells proliferate inappropriately, albeit temporarily, in the absence of *p57* (this work; Zhang et al. 1997). In contrast, *p27* is normally not required for lens development but contributes significantly to cell cycle arrest and differentiation in the absence of *p57*. Although these inhibitors are expressed

at the highest levels in the equatorial zone, they are also expressed at low levels throughout the lens. Persistent expression of these two *CKI*s is necessitated by the continued presence of D-type cyclins in the developing lens (Fig. 1A–C), which also helps explain the unscheduled S-phase entry in the absence of these inhibitors. The inability of lens fiber cells to undergo cell cycle arrest leads to defects in differentiation, including elongation and β/γ-crystallin expression, which are more severe in *p57/p27* double mutants than in *p57* single mutants.

The phenotypes observed in *p27/p57* mutant lenses are reminiscent of those seen in *Rb*-deficient lenses (Morgenbesser et al. 1994), consistent with the biochemical roles of *CKI*s as activators of *Rb*. Because hypophosphorylated *Rb* plays a critical role in differentiation, it is likely that the inability of lens fiber cells to differentiate in *p27/p57* mutants reflects increased *Rb* phosphorylation and inhibition of its differentiation-promoting function. However, two significant differences exist between the phenotypes of the *Rb* versus *p27/p57* double mutants. First, the extent of overproliferation as assessed by BrdU incorporation appears to be significantly greater in *p27/p57* mutants than in *Rb* mutants. This may reflect the fact that these two *CKI*s function not only upstream of *Rb* by blocking cyclin D/*Cdk4* activity but also function downstream of *Rb* by blocking cyclin E/*Cdk*2-mediated S-phase entry. Alternatively, the increase in *Cdk* activity due to CKI loss may result in inactivation of additional *Rb*-family members such as p130 and p107, thereby producing a more severe proliferation defect than *Rb* loss alone. Thus, proliferation of lens fiber cells lacking *Rb* may be limited because of the action of *p27* and *p57* on *Cdk*s. The second major difference is that the rates of apoptosis in CKI-deficient lenses are much lower than those in *Rb*-deficient lenses and are similar to the rates seen in *Rb*/p53 double mutant lenses. *Rb* is required to establish the transcriptional program that brings about differentiation of multiple cell types but has also been shown to inhibit apoptosis during myoblast differentiation (Wang et al. 1997) and in other situations (for review, see Wang 1997). Thus, low rates of apoptosis in *p27/p57*-mutant lenses may reflect an antiapoptotic role for *Rb*. If the absence of *p27* and *p57* result in the inactivation of *Rb* to such an extent that it phenocopies the differentiation defect of the *Rb* null mutant lenses, why the difference in apoptosis rates? There are several plausible explanations for this difference. First, *Rb* could have an antiapoptotic function that is not regulated by *Cdk* phosphorylation and therefore would not be altered by CKI loss. Second, even in the absence of the *CKI*s, there may be residual *Rb* activity such that apoptosis-inhibiting functions of *Rb* are largely intact. Even in the absence of *CKI*s, there is likely to be residual regulation of *Rb* if *Cdk* activity is still cyclical. In contrast, an *Rb* null mutant cell would constituively derepress all *Rb*-regulated genes such as E2F1, an apoptosis-inducing gene (Qin et al. 1994; Shan and Lee 1994; Kowalik et al. 1995) and might display a more severe phenotype for this reason. Third, it is also possible that CKI mutant cells have higher *Cdk* activity levels and these act to prematurely inactivate E2F1 function (Dynlacht et al. 1994; Krek et al. 1994), thereby balancing the apoptotic-inducing consequences of inactivating *Rb*. The fact that the apoptosis rates of the *p27/p57* double mutants are similar to the rates observed in the *Rb*/p53 double mutant mice (Morgenbesser et al. 1994) is consistent with interfering with E2F1 function because apoptosis caused by *Rb* loss is partially mediated by E2F1 (T. Jacks, pers. comm.) and E2F1-mediated apoptosis is p53-dependent (Qin et al. 1994; DeGregori et al. 1997).

*CKI*s are the ultimate effectors of signal transduction pathway intended to bring about cell cycle arrest, and the patterns of expression during embryonic development suggest that particular *CKI*s play important roles in terminal differentiation in a tissue-specific manner. However, the fact that mice lacking single *CKI*s display surprisingly few developmental phenotypes has brought into question the essential nature of *CKI*s for cell cycle arrest and differentiation. Our results demonstrate that two *CKI*s, *p57* and *p27*, cooperate to control proliferation and differentiation in multiple tissues and reiterate the critical importance of *CKI*s to cell cycle control during development.

Materials and methods

Genotypic analysis

Mice deficient for *p57* and *p27* (Δ-51 allele) have been described (Kiyokawa et al. 1996; Zhang et al. 1997). The original *p57* knockout was made in AB1 ES cells derived from a substrain of the 129 mouse, 129SvEvBrd–Hprt^{b-m2}, whereas the *p27* knockout was in the ES line CJ7, derived from another substrain of 129 mouse, 129Sv. These two 129 substrains are quite different according to Simpson et al. (1997). Thus, the original *p57* knockout resides in a background hybrid between C57BL/6 and 129SvEvBrd–Hprt^{b-m2}, and the current cross produced double mutants in a mixed background of C57BL/6, 129Sv, and 129SvEvBrd–Hprt^{b-m2}. We have developed PCR protocols to identify wild-type and disrupted alleles of *p27* and *p57*, using a set of three primers for each gene. For *p27*, the sequences of the primers are primer 1, ACGTGAGAGTGTCTAACGG; primer 2, AGTGCTTCTCCAAGTCCC; and primer 3, GCGAGGATCTCGTCGTGAC. For *p57*, the sequences of the primers are primer 1, CGTCCACAGGCCGAGTGC; primer 2, GCTGCGGAGGTACACGTCG; and primer 3, GCGAGGATCTCGTCGTGAC. Detailed protocols are available upon request.

Phenotypic analysis

Embryos were processed using standard histological procedures. β- and γ-crystallins were detected using polyclonal antibodies provided by K. Mahon (Baylor College of Medicine, Houston, TX) and visualized with FITC-conjugated secondary antibody (Amersham). For cell proliferation assays, pregnant mice were injected with BrdU (0.1 mg/gram body weight) 2 hr prior to delivery by cesarean section. S-phase cells were visualized using an anti-BrdU monoclonal antibody (Dako) in conjunction with an FITC-conjugated secondary antibody (Amersham). In situ hybridization was performed as described (Matsuoka et al. 1995; Parker et al. 1995). Probes for D-type cyclins were provided by C. Sherr (St. Jude Children's Research Hospital, Memphis, TN). Apoptotic cells were detected with a kit from Trevegene, and assays were performed as recommended by the manufacturer.

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